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# **PROCEDURES AND APPROVED METHODS FOR CONDUCTING BRUCELLA RING TESTS**

## **B.R.T.**

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ANIMAL DISEASE ERADICATION DIVISION  
AGRICULTURAL RESEARCH SERVICE  
U.S. DEPARTMENT OF AGRICULTURE

## PREFACE

The Brucellosis Ring Test (BRT) has been an integral part of the Brucellosis Eradication Program in the United States for about 10 years.

The acceptance of the BRT by cooperating States and the Animal Disease Eradication Division, Agricultural Research Service, United States Department of Agriculture, has afforded a twofold advantage: (1) detection of brucella infection in lactating herds; (2) surveillance of brucellosis eradicating progress that has been accomplished.

Records and data processing of the BRT results are essential. The ADE Division has treated the record-keeping subject in the Manual on Program Records.

This revised edition of the BRT Manual includes methods and techniques for collection of samples, application of tests, and interpretation of results. Maintaining uniformity in conducting the BRT is a responsibility of all participating agencies in cooperation with the ADE Division of the Agricultural Research Service.

As new techniques are developed, supplemental instructions will be issued to replace sections of this manual.

Animal Disease Eradication Division  
Technical Services

REVISED JANUARY 1965

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## PROCEDURES AND APPROVED METHODS FOR CONDUCTING BRUCELLA RING TESTS

### B.R.T.

#### Central or Stationary Area Laboratories

Originally, mobile laboratories were used in the inauguration of the BRT program and were an educational adjunct to the accelerated brucellosis program, but now their use is confined to a few localities.

The State-Federal Cooperative Laboratories have proven to be the most practical place to conduct the BRT in most instances.

#### Milk Collections

Methods of handling milk change with industrial and mechanical advancements; thus alterations in BRT milk sampling will become necessary. The technicians collecting the milk or cream samples should have full understanding of these methods and should be assured that the identity of the sample is maintained and that mixing, or "carry over" from one patron to another, is minimized.

The State Dairy Divisions may provide lists of plants purchasing milk and cream. The Agricultural Extension Service and the County Health Departments furnish valuable information on other sale points of dairy products in the area.

#### Sampling fresh milk from "can" delivery

The collection of fresh milk or cream should be made by personnel authorized by the State-Federal regulatory officials rather than by the patrons. A milk sample should be collected from each can offered to the plant on that particular day of delivery. No more than three cans of milk should be pooled into one collection tube. When cans of milk are allowed to stand and cream rises to the surface, care is necessary to avoid collecting excess cream in the sample.

If collection is made from the can, a tin-coated metal dipper with a 12-inch handle should be used to thoroughly agitate and collect the milk. The design of the dipper allows for a washing effect to free it from excess surface cream when taken as deep in the can as possible.

In another method, the collection is made when the can is placed in the cradle and the contents are transferred to the weighing vat. The metal collection dipper may be used to collect the milk sample from the stream of milk coming from the can. By passing the dipper through the stream from beneath the can, and away from the stream, the collector can obtain a milk sample with a normal cream content.

The metal collection dipper, held with the handle in an upright position, holds 3 cc. When a sample from no more than three cans is placed in the vial, the collection tube should have an appreciable airspace. After all cans belonging to one patron have been sampled, the dipper is rinsed free of milk in fresh warm water before it is used again.

### Babcock milk

The State-Federal regulatory officials should enlist the wholehearted cooperation of the State Dairy Division and encourage local rules and regulations at the State level to be formulated to insure the application of the following plant techniques:

1. Bulk or Weighing Tank. - Satisfactory mechanical or stationary equipment should be so designed as to provide for the collection of statistically accurate pooled samples.
2. Responsibility of Person Taking the Sample. - Every effort should be made to assure that samplers are familiar with the importance of proper sampling techniques.
3. Method of Taking Milk Samples. - The dipper method is most commonly used for maximum uniformity of quantity and freedom from "carry over" from preceding use. Routine rinsing of dipper in new pooled milk or suitable diluent should be practiced.
4. Amount of Milk Sample Taken. - A minimum of 10 ml. of milk should be taken for daily collection of a biweekly Babcock test. When alternate daily collections are made, 20 ml. should be taken, in order that a minimum of 140 ml. is available for the semimonthly Babcock test.
5. Suitable Preservative of Composite Sample. - Red tablets containing mercuric bichloride compound are widely used as an efficient preservative. One 0.5 gram ( $7\frac{1}{2}$  grain) tablet is sufficient for a semimonthly composite collection of 4 or more ounces (see 4) when refrigerated storage is available.
6. Size of Collection Bottle for Preserved Composite Samples. - The 8-ounce-capacity sample bottle is the most desirable size for collection (see 4 and 5). A tight-fitting stopper attached to the bottle, preferable of rubber, carrying the identification number of the patron selling the milk is satisfactory.
7. Mixing Daily Additions. - Each sample collection of fresh milk should be mixed into the preserved portion by gentle rotation or complete inversion of the capped bottle.
8. Storage of Preserved Composite Samples. - Refrigerated storage



at 40°-50°F. is recommended for preserved composite samples when not actually being used.

9. Temperatures of Composite Sample for Fat Determination. - Heating preserved composite milk samples to temperatures of 91°-100°F. in a relatively short time (30 minutes) is most desirable. The water bath in which samples are heated should have an automatic temperature control capable of maintaining 105°-110°F. to avoid overheating, which will destroy BRT testing qualities.

10. Holding Samples After Fat Determination. - It is recommended that preserved composite milk samples be held in refrigeration at 40°-50°F. for 10 days, subject to call. The BRT sample should be collected as soon as permissible.

The BRT sample can be collected by either of the following methods. With either method, it is imperative that the cream on the preserved composite sample be well dispersed.

Method 1. - The cold Babcock samples bottle should be held at a 40° angle and should be resting on a flat tabletop surface; in this position, it should be rotated rapidly at least 10 times in a 12-inch circle. Approximately 5 ml. of the milk should be transferred to a suitable collection tube.

Method 2. - Transfer of 5 ml. of preserved sample may be made from heated bottle under controlled temperatures at the time the butterfat test is conducted (see Step 9).

#### Fresh Milk From Farm Holding Tanks for BRT

In many instances, especially from the larger milksheds for Grade "A" milk, it is advantageous to collect fresh milk from the milk tank. The tank-truck operator collects fresh samples from the farm holding tank in a plastic bag or bottle that is identified with the route, patron number, and date. The samples are placed in a refrigerated compartment on the truck and delivered to the plant, receiving station, or cooperative. Representative milk samples may be obtained from these Stations or from a State or City-County health department.

NOTE: Consider that bacteriological tests, etc., will be conducted at frequent intervals on Grade "A" milk from all patrons in an area by a health department (either State, City-County, or private laboratories on contract). Such samples are kept under conditions that afford good quality. Records with regard to patrons' names and addresses are usually in excellent order; and, because several plants' bacteriological tests may be conducted in one laboratory, collection by BRT technicians may be simplified.

## Cream Collections

It is believed the most practical means of collecting cream for a BRT must be considered in the interest that serves each area best. A sample of cream should be taken from each cream can and placed in a separate tube regardless of the number of cans a producer may have.

## Formaldehyde Preservative for Fresh Milk

Formaldehyde may be used as a preservative at the time of fresh milk collections. Such a solution is made by adding 1 ounce of 37 percent formaldehyde (the commercial grade) to a gallon of distilled water. One-half ml. preservative solution should be added to the collection tube prior to milk collection.

## Setting up the BRT on Milk

It is desirable to allow all samples taken directly from a can or farm holding tank to "age" under refrigeration before conducting the test. The aging period should be at least 48 to 72 hours.

The milk and antigen should be removed from the refrigerator and held at room temperature at least 1 hour prior to setting up the test. It is not considered a good practice to warm up milk in a water bath prior to mixing. Any heating of the samples has a tendency to destroy antibodies that may be present.

The milk in the collection tube should be gently shaken to insure thorough mixing of cream and skim portions; 1 ml. of milk should be transferred to a separate test tube to which 1 drop (0.03 ml. from the standard BRT dropper) of BRT antigen is then added, and the mixture inverted several times to insure proper mixing of antigen and milk.

Delivery of uniform drops of antigen can be made accurately at a rate of 40 to 50 drops per minute. Care should be taken to hold the dropper in a vertical position and to avoid shaking the drops off the delivery tip.

## Incubation Time and Temperature

Incubation of milk that has been collected directly from cans should be 30

minutes at 37.5°C. This milk may or may not contain formaldehyde preservative.

Samples obtained from farm holding tanks and from composite collection bottles (whether from cans or tanks) should be incubated 1 hour at 37.5°C. Immediately after mixing the milk and antigen, the rack should be placed in an incubator until reading time.

The temperature-controlled heating cabinet should be turned on an hour before use. The heating unit placed at the bottom is controlled by the thermostat, which should be adjusted to 37.5°C. ( $\pm 1^\circ\text{C}$ .). A thermometer must be used in the escape ventilation holes at the top to adjust the thermostat to the desired temperature.

### Modified Cream Ring Test

A modification of the cream ring test has been devised that increases the sensitivity over the previous test. This test has the advantage of detecting a greater number of infected herds producing cream.

The test is most efficient when applied to good-quality cream specimens. The cream antibody content is affected by adverse factors such as:

1. the hydrogen ion concentration below pH 5.0
2. collection periods exceeding 2 weeks
3. collection and storage periods combined exceeding 2 weeks

The periods of time indicated can only be used as guidelines. Other factors should be considered such as refrigeration and local conditions.

### Procedures

1. Add precollection diluent<sup>1</sup> (approximately 0.6 ml. diluent to 4 ml. cream) to each tube before cream is collected.
2. Keep cream refrigerated until test is to be conducted.
3. At the time of test, warm the cream specimens to room temperature.

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<sup>1</sup> Precollection diluent consists of:

Saturated sodium bicarbonate . . . . .	20 oz.
formalin . . . . .	1 oz.
tapwater q.s. . . . .	1 gallon



4. Centrifuge cream at approximately 1,000 g. for 15 minutes (in the 14 x 100 mm. collection tubes).
5. Observe after centrifugation. Those appearing white (like skim milk) are classified as not sour. Those appearing colorless to slightly yellow are classified as sour. Record this information.
6. With a 15 ga. 4-inch cannula attached to a 2 ml. rheometer syringe, set the delivery at 1.2 ml. With the plunger depressed, insert the cannula through the fat layer and withdraw from the liquid portion. Both fat and skim solids interfere with the test.
7. Add this liquid portion to 0.6 ml. modified cream ring neutralizer<sup>2</sup> in a 14 x 100 mm. tube. Clean the syringe and repeat on remaining specimens.
8. Thoroughly mix the cream and neutralizer.
9. Add 0.4 ml. of fresh negative raw cream, pooled from 20 or more cows, with a rheometer syringe. Keep the pooled cream refrigerated while not in use.
10. Add 1 drop (0.03 ml.) of BRT antigen to each tube.
11. Mix contents.
12. Incubate 1 hour at 37°C.
13. Observe and record results.

#### Reading the Milk and Cream BRT Tests

A uniform light source is necessary; excessive light as from the sun or suppressed light on dark days should be avoided. It has been found that a fluorescent lamp providing 35 to 50 foot-candles of blue-white is

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<sup>2</sup> Modified cream ring test neutralizer:

- a. Make saline by weighing 8.5 gr. of NaCl and adding water to make 1,000 ml.
- b. Saturate the saline with sodium bicarbonate.
- c. Mix a portion of this solution with an equal part of evaporated milk.

effective. For this purpose, the lamps should be suspended above the operator's head and within 18 to 22 inches of the samples to be read. A fluorescent lamp with side reflectors using two 20-watt daylight tubes (bluish color) is desirable to produce a shadow-free light source and prevent eyestrain.

### Interpretation of BRT Results

#### A. Milk samples from cans incubated 30 minutes.

The ring test in which the intensity of color in the cream layer is deeper than that in the skim portion is "suspicious". All others are "negative".

#### B. Milk and cream samples incubated 1 hour.

The ring test in which any detectable blue ring or accumulation of blue color in the cream layer at the top of the milk column is present should be considered "suspicious". All others should be considered "negative". All milk and cream ring test results may be recorded as "N" or "S" to denote negative or suspicious.

Observation should be made to note loss of blue color from both skim and cream portions of milk at time of reading. An unsatisfactory test has been conducted when the increased acidity of sour cream sample inhibits the detection of dye stained antigen.

#### CAUTION:

When tests disclose an insufficient cream layer by the presence of a thin blue line at the top of the test column, a retest is recommended. The retest can be made by resetting the sample as in the regular test with the addition of 4 to 6 drops of known negative cream just prior to the addition of antigen. Negative cream for this purpose may be recovered from fresh (raw) negative milk samples that have not been heated to incubation temperatures. Recovery of cream from preserved samples is not recommended. Local arrangements should be made to have sufficient fresh whole milk available for laboratory needs. This auxiliary cream should be obtained from pooled negative milk in which at least 20 cows are represented. This is essential to eliminate inherent errors often found when the BRT is applied to individual animals.

### Factors Influencing the BRT

Technicians collecting and testing milk samples should be aware of the factors that may alter the Brucella antibody content of milk from infected cows.

1. Concentration of Mercuric chloride. - This preservative, when used as recommended (i.e., one 0.5 gram ( $7\frac{1}{2}$  grain) tablet per 140 ml. of milk), causes very little loss in Brucella antibodies. Excessive mercuric chloride concentrations adversely affect the detection of antibody in milk.
2. Reheating of the Babcock sample. - If the heating of Babcock sample exceeds 110°F. for over 5 minutes, the antibody content decreases.
3. Time and Temperature of Storage. - Composite milk samples stored at 45°F. will retain sufficient Brucella antibody for testing for about 3 weeks. Higher temperatures and longer periods of storage causes greater loss of antibody.
4. Ratio of Milk and Antigen used in the Test. - The standard procedure is 1 drop (0.03 ml.) of antigen to 1 ml. of milk. Variations from this will vary the test results, i.e., with increase in quantity of milk the sensitivity increases and, conversely, with increase in quantity of antigen the sensitivity decreases. Therefore, antigen droppers should be adjusted to deliver 0.03 ml. per drop and accurate measurement of milk is essential.
5. Nonspecific Reactions (or False Suspicious Reactions). -
  - (a) Fresh milk tested the day drawn will occasionally show a suspicious reaction that will disappear after refrigeration.
  - (b) Milk from cows with mastitis occasionally shows a suspicious reaction to the BRT.
  - (c) Colostrum and milk from the cow "drying up" usually shows a suspicious reaction to the BRT.

A false suspicious reaction is defined, here, as a reaction to the BRT with a negative agglutination blood serum test.

Experience has shown that the milk antibody system and the blood serum antibody system function independently of each other. Thus, for example, occasionally Brucella antibodies may be detected in the milk before the blood serum antibodies are apparent in a low-grade udder infection.





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